the phenotypic heterogeneity of nevi spili.

HRAS mutations have recently been demonstrated in a number of hamartomatous cutaneous neoplasms including epidermal nevi and nevus sebaceous (Groesser *et al.*, 2012; Hafner *et al.*, 2012; Levinsohn *et al.*, 2012; Sun *et al.*, 2013). When these alterations occur early in development or affect multiple cell lineages, they can result in developmental syndromes called 'RASopathies,' such as Schimmelpenning, epidermal nevus syndrome, and phacomatosis pigmentokeratotica (Hafner and Groesser, 2013). The *HRAS* point mutation (c.37G->C, p.Gly13Arg), in particular, appears to be overrepresented among *HRAS* mutations in these cutaneous congenital conditions. This mutation allows constitutive activation of RAS pathway likely facilitating the development of secondary neoplasms through accrual of additional genetic alterations.

Our finding adds sporadic nevus spilus to the spectrum of congenital cutaneous lesions that harbor activating mutations in *HRAS*. In nevus spilus, we speculate that the melanocytic neoplasms that arise from these tan patches likely acquire additional genetic alterations that enable progression. Indeed, we have recently demonstrated such a secondary change through amplification of *HRAS* in Spitz nevi arising out of a nevus spilus (Sarin *et al.*, 2013). However, the secondary mutations that give rise to the diverse spectrum of melanocytic neoplasms have not yet been identified. The knowledge of the genetic basis of nevus spilus represents a further step towards understanding the genetic etiology underlying melanocytic neoplasms. In addition, these lesions represent a unique opportunity to study the genetic alterations that allow progression from a nevus spilus to a nevus or melanoma in the setting of constitutive RAS/MAPK activation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Decreased Expression of the Chromatin Remodeler ATRX Associates with Melanoma Progression

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TO THE EDITOR

ATRX is a member of the SWI/SNF family of chromatin remodelers, originally identified as mutated in patients with Alpha Thalassemia/Mental Retardation, X-linked syndrome. The protein product contains several highly conserved domains, including an ADD (ATRX-DNMT3-DNMT3L) domain that binds methylated histone H3 at lysine 9 and an ATPase domain responsible for its remodeling activities (Ratnakumar and Bernstein, 2013). Recently, whole-genome sequencing studies identified ATRX mutations in multiple tumors, including those of neural crest cell origin: neuroblastoma, low-grade glioma, and glioblastoma (Heaphy *et al.*, 2011a; Jiao *et al.*, 2011; Cheung *et al.*, 2012;

Abbreviations: ATRX, alpha thalassemia/mental retardation, X-linked; IHC, immunohistochemistry

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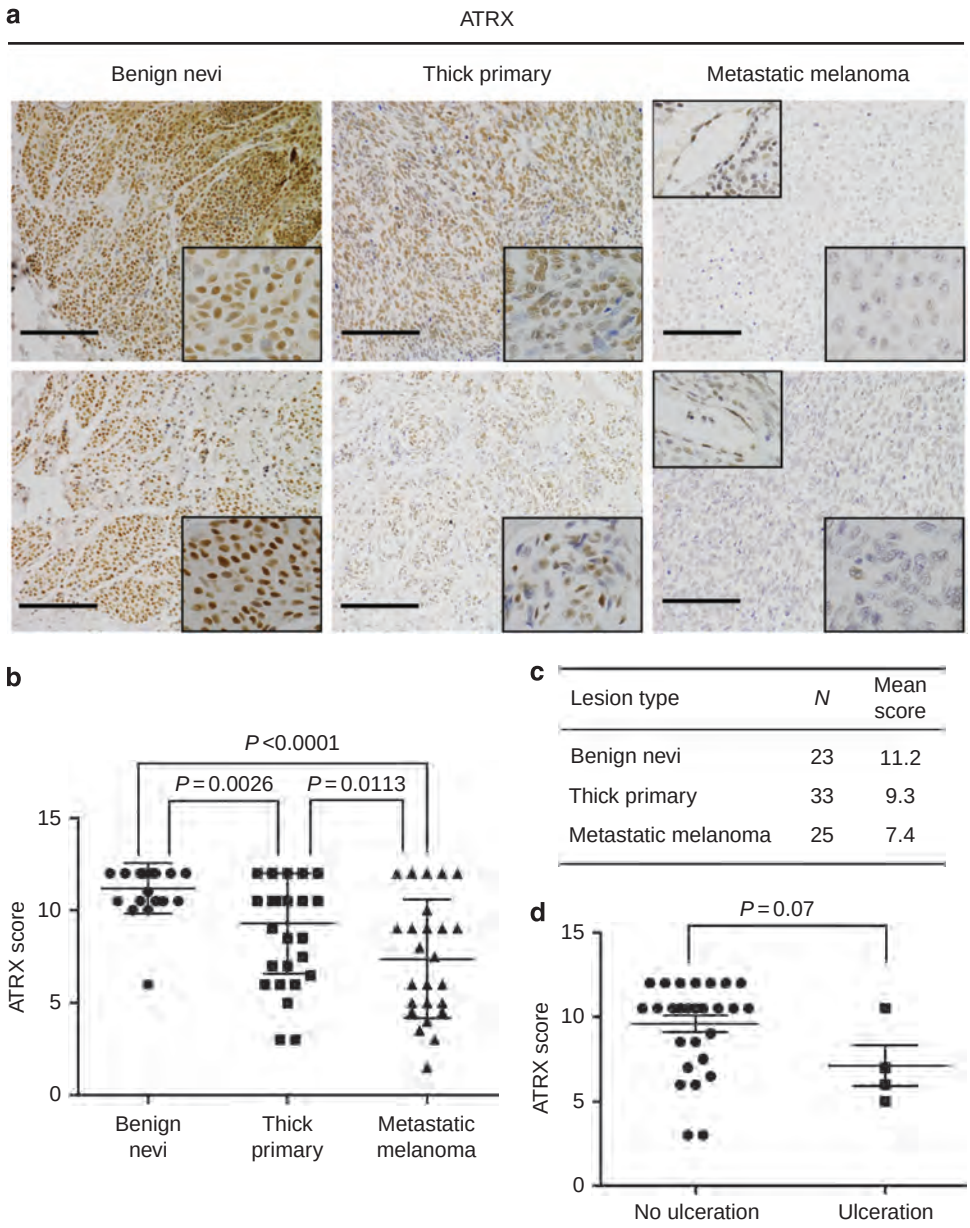

Figure 1. Loss of ATRX protein expression is associated with melanoma progression. (**a**) Immunohistochemistry (IHC) for ATRX in representative benign nevi, thick primary, and metastatic melanoma tissue. Images were taken at ×20 magnification; insets (bottom right) show nuclei at ×40 magnification. Insets (top left) show an ATRX-positive stain of endothelial cells within metastatic specimens. Bar = 100 μm. (**b**) IHC scores of benign nevi and thick primary and metastatic melanoma from two independent pathologists. Each tissue section was quantified based on number of positively stained cells (1–4) multiplied by stain intensity (1–3) to generate a score. (**c**) Table summarizing number of total samples and average IHC score per lesion. (**d**) ATRX protein levels versus presence of ulceration in primary melanoma specimens. All statistical significance assessed using the two-sided Mann–Whitney *U*-test, *P*-value indicated. Mean ± SD.

Kannan *et al.*, 2012; Schwartzentruber *et al.*, 2012). ATRX alterations encompass point mutations throughout the coding region as well as large N-terminal deletions. Although mechanistically unclear, ATRX mutations result in loss of protein as assessed by immunohistochemistry (IHC) and often correlate with alternative lengthening of telomeres (ALT) (Heaphy *et al.*, 2011a; Cheung *et al.*, 2012; Kannan *et al.*, 2012; Schwartzentruber *et al.*, 2012).

To our knowledge, an investigation of ATRX in cutaneous melanoma is currently lacking. Our previous studies have demonstrated that decreased expression of histone variant macroH2A drives melanoma cell proliferation and metastasis (Kapoor *et al.*, 2010), and that ATRX interacts with macroH2A to negatively regulate its association with chromatin (Ratnakumar *et al.*, 2012). Taken together with recent reports of decreased ATRX protein in neural crest cell–derived tumors, we hypothesized that ATRX function might be compromised in melanoma.

In order to test this hypothesis, we performed IHC on a panel of 23 benign nevi, 33 primary melanoma (⩾1.0 mm

deep), and 25 metastatic melanoma specimens that were formalin fixed and paraffin embedded (FFPE) (Figure 1a and c). Slides were evaluated by two dermatopathologists in a blinded fashion using a scoring system based on number of positive nuclei and staining intensity (inter-rater correlation $r=0.693$, $P<0.0001$; see Supplementary Methods online for details). As depicted in Figure 1a, ATRX protein expression is appreciably reduced with increased malignancy. Benign nevi showed a higher proportion and intensity of nuclear staining when compared with metastatic lesions (Figure 1a and b; $P<0.0001$). Furthermore, ATRX protein expression was reduced between benign nevi and primary melanoma, with heterogeneous staining observed in the latter ($P=0.0026$; Figure 1a and b), and between primary and metastatic melanoma ($P=0.0113$; Figure 1b). This suggests a potential stepwise loss of ATRX expression during melanoma progression.

We further examined whether ATRX levels in primary melanoma correlated with clinicopathological predictors of prognosis. ATRX staining did not correlate with depth of the lesion (data not shown); however, the primary melanomas examined in our cohort were of a Breslow thickness >1.0 mm (average depth, 5.6 mm), and thus were quite aggressive. We did, however, find an inverse correlation with the presence of ulceration, a poor prognostic factor (Figure 1d). Because our study is

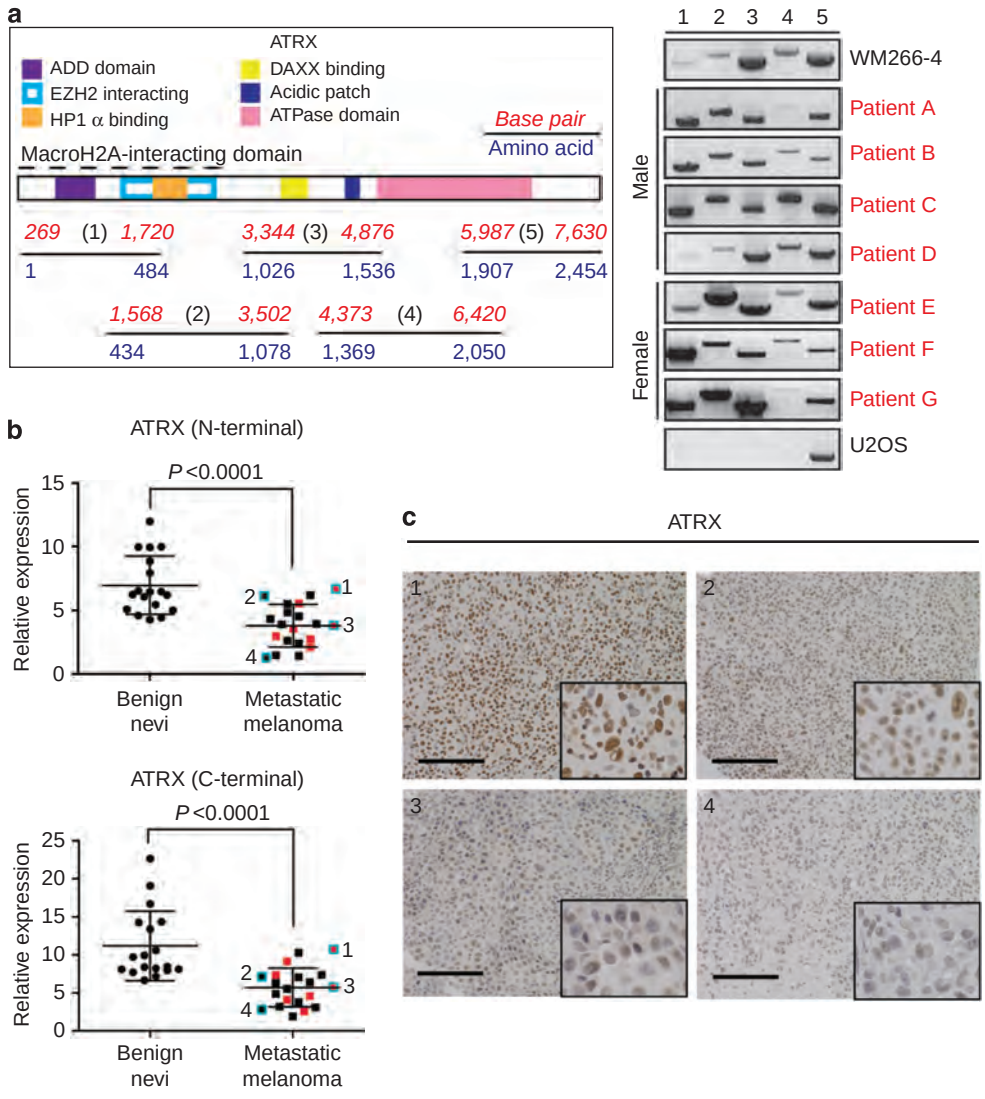

Figure 2. ATRX mRNA levels are decreased in metastatic melanoma. (**a**) Illustration of ATRX with domains and five amplicons depicted (left). ATRX cDNA was amplified as indicated for analysis of putative deletions in metastatic melanoma specimens (right). WM266-4 and U2OS were used as controls. (**b**) qRT-PCR analysis of ATRX from fresh frozen benign nevi and metastatic melanoma lesions. N- and C-terminal primers were used. Melanoma specimens analyzed in **a** are depicted in red and in **c** are highlighted in blue and numbered. Expression levels were normalized to GAPDH and statistical significance was derived using unpaired Student's *t*-test, *P*-values indicated. Mean ± SD. (**c**) ATRX IHC in representative metastatic melanoma tissues from **b**. Images were taken at ×20 magnification; insets (bottom right) show nuclei at ×40 magnification. Bar = 100 μm.

retrospective with a small sample size, we note that any correlations, or lack thereof, are preliminary.

Because structural variations of ATRX exist in neuroblastoma and osteosarcoma (Cheung *et al.*, 2012; Lovejoy *et al.*, 2012), we determined whether such alterations are present in metastatic melanoma. Using a technique to detect structural variations of ATRX, we performed qualitative reverse transcriptase (RT)–PCR of cDNA derived from a cohort of fresh frozen metastatic melanoma samples ($n=7$). Owing to the large ATRX-coding region, we amplified the cDNA into five fragments ranging from 1.5 to 2 kb pairs. Because ATRX is located on the X chromosome, we analyzed both male and female patients for potential effects due to gene dosage. Our analysis shows that the ATRX gene product is intact in all metastatic melanomas assayed, as evidenced by appropriately sized bands within each sample (Figure 2a). The cell line WM266-4 derived from a melanoma metastasis served as a positive control for PCR amplicons, as it is devoid of ATRX mutations (Cancer Cell Line Encyclopedia at http://cbioportal.org). The osteosarcoma cell line U2OS, which has large deletions of the ATRX locus (Lovejoy *et al.*, 2012), was used to ensure that our assay worked effectively. This analysis suggested that decreased ATRX protein levels in metastatic melanoma is unlikely to be the result of large genomic alterations.

We next queried whether diminished ATRX protein in metastatic disease was due to transcriptional regulation. We performed qPCR analysis on a cohort of 18 fresh frozen benign nevi and 20 metastatic melanoma tumors, including those samples analyzed for deletions (Figure 2a; indicated in red in Figure 2b). Using both N- and C-terminal primers for ATRX, we found a statistically significant loss of ATRX mRNA levels in metastatic melanoma as compared with benign tissue ($P<0.0001$; Figure 2b). We next performed IHC on a subset of these tumors, for which FFPE tissue was available (indicated in blue in Figure 2b). The level of ATRX protein indeed corroborated our qPCR findings (Figure 2c). Collectively, these results indicate that ATRX loss occurs, at least in part, by transcriptional repression resulting in loss of protein expression in late-stage disease.

Collectively, we demonstrate that ATRX loss correlates with melanoma progression. Using two independent cohorts (FFPE and fresh frozen; total of 119 tissues), we found a significant decrease of both mRNA and protein levels of ATRX in metastatic melanoma. Although it remains to be tested in a prospective study, ATRX may serve as a biomarker to predict prognosis of disease. Although we did not find evidence of large genomic alterations in a subset of melanoma patients, we do not exclude the possibility of ATRX mutations in melanoma. In fact, a 4–7.5% rate of mutation in cutaneous melanoma is reported by TCGA, Broad and Yale studies (http://www.cbioportal.org). Interestingly, these mutations are distributed throughout the ATRX-coding region and do not correlate with decreased mRNA levels (Supplementary Figure S1 online). This suggests that multiple mechanisms underlie ATRX dysregulation in melanoma—transcriptional regulation as described here, and point mutations that may result in loss of protein expression, as reported for other tumor types (Cheung *et al.*, 2012; Kannan *et al.*, 2012; Schwartzentruber *et al.*, 2012).

Although ATRX staining did not anti-correlate with macroH2A levels (data not shown), we previously showed that macroH2A is transcriptionally silenced by DNA methylation in malignant melanoma and thus might not be regulated at the level of chromatin deposition (Kapoor *et al.*, 2010). The mechanism by which ATRX transcription is suppressed in melanoma may also be through epigenetic silencing (e.g., DNA methylation or histone modifications), or by microRNA-mediated regulation (Pacurari *et al.*, 2013). Finally, we posit that investigating the chromatin landscape of tumors that have lost ATRX expression should provide insights into the mechanism(s) by which ATRX loss drives melanoma progression.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Transplantable Malignant Melanoma in LT.B6 Congenic Mice Resembling Pigmented Epithelioid Melanocytoma in Humans

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TO THE EDITOR

The transplantable B16 melanoma model has been used for decades and continues to be used with various degrees of reproducibility in mice (Fidler, 1975). Melanocytic tumors or nevus-like lesions were induced in two-stage cutaneous chemical oncogenesis experiments in various inbred strains of mice (Bannasch and Goessner, 1994; Sundberg *et al.*, 1997; Maronpot *et al.*, 1999). Recently a number of genetically engineered mouse models have been generated to recapitulate the major signaling pathways deregulated in human melanoma, namely, the RAS–RAF–MAPK, PI3K–AKT, CDK4–INK4A–RB1, and ARF–TP53 pathways (reviewed in the study by Damsky and Bosenberg (2010) and Walker *et al.* (2011)). These preclinical models have been invaluable to delineate the relationship between causative gene mutations and molecularly targeted therapeutics; however, there are no spontaneously occurring melanocytic tumors in laboratory mice to globally discover other disrupted gene networks. Although melanomas are relatively common in humans and domestic animals exposed to sunlight, the scarcity of spontaneous melanomas in laboratory mice might be the result of the mice never being exposed to sunlight or artificial UVR under normal husbandry conditions. Herein, we report the finding of a spontaneous, locally invasive, transplantable malignant melanoma that resembles pigmented epithelioid melanocytoma (PEM), formally known as the ''animal/equine-type'' in humans (Zembowicz *et al.*, 2004).

We found a 172-day-old female LT.B6 ''line E'' congenic mouse on routine examination to have multiple raised black nodules on the tail (Figure 1a). Histopathology showed diffusely and heavily pigmented dermal

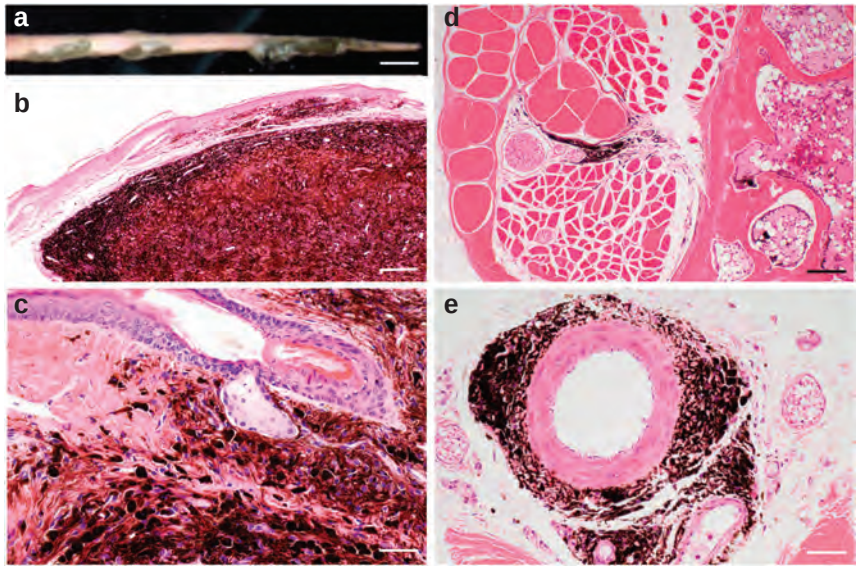

Figure 1. Clinical and histopathological features of LT.B6 congenic mouse. Multiple black nodules were noted on the tail of a 172-day-old female mouse (**a**), scale bar = 7 mm; histologically showing heavily pigmented dermal nodules (**b**), scale bar = 200 μm; sparing the adnexae (**c**), scale bar = 50 μm; surrounding but not invading the tail nerves (**d**), scale bar = 100 μm or the ventral coccygeal artery (**e**), bar = 50 μm.

Abbreviations: CT, cycle threshold; LN, lymph node; Mitf, microphthalmia-associated transcription factor; qRT-PCR, quantitative real-time reverse transcriptase–PCR; PEM, pigmented epithelioid melanocytoma; Tyrp1, tyrosine-related protein 1

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